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The antimicrobial and antibiofilm effects of gentamicin, imipenem, and fucoidan combinations against dual-species biofilms of *Staphylococcus aureus* and *Acinetobacter baumannii* isolated from diabetic foot ulcers

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Abstract

Introduction Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia due to impaired insulin production or utilization, leading to severe health complications. Diabetic foot ulcers (DFUs) represent a major complication, often exacerbated by polymicrobial infections involving *Staphylococcus aureus* and *Acinetobacter baumannii*. These pathogens, notorious for their resistance to antibiotics, complicate treatment efforts, especially due to biofilm formation, which enhances bacterial survival and resistance. This study explores the synergistic effects of combining gentamicin, imipenem, and fucoidan, a sulfated polysaccharide with antimicrobial properties, against both planktonic and biofilm forms of *S. aureus* and *A. baumannii*.

Methods Isolates of *S. aureus* and *A. baumannii* were collected from DFUs and genetically confirmed. Methicillin resistance in *S. aureus* was identified through disk diffusion and PCR. Biofilm formation, including dual-species biofilms, was analyzed using the microtiter plate method. The antimicrobial efficacy of gentamicin, imipenem, and fucoidan was assessed by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC). Synergistic interactions were evaluated using the fractional inhibitory concentration index (FICi) and fractional bactericidal concentration index (FBCi). The expression of biofilm-associated genes (*icaA* in *S. aureus* and *bap* in *A. baumannii*) was analyzed, and the cytotoxicity of fucoidan was assessed.

Results The study revealed that 77.4% of *S. aureus* and all *A. baumannii* isolates showed multidrug resistance. Among 837 tested conditions for dual-species biofilm formation, 72 resulted in strong biofilm formation and 67 in moderate biofilm formation. The geometric mean MIC values for gentamicin were 12.2 µg/mL for *S. aureus*, 22.62 µg/mL for *A. baumannii*, and 5.87 µg/mL for their co-culture; for imipenem, they were 19.84, 9.18, and 3.70 µg/mL, respectively, and for fucoidan, 48.50, 31.20, and 19.65 µg/mL, respectively. The MBC values for gentamicin were 119.42, 128,

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and 11.75 µg/mL; for imipenem, they were 48.50, 14.92, and 8 µg/mL; and for fucoidan, they were 88.37, 62.62, and 42.48 µg/mL. The MBIC values were 55.71, 119.42, and 18.66 µg/mL for gentamicin; 68.59, 48.50, and 25.39 µg/mL for imipenem; and 153.89, 101.49, and 53.53 µg/mL for fucoidan. The MBEC values were 315.17, 362.03, and 59.25 µg/mL for gentamicin; 207.93, 157.58, and 74.65 µg/mL for imipenem; and 353.55, 189.46, and 99.19 µg/mL for fucoidan. When cultured in planktonic form, the geometric mean FICi and FBCi values indicated additive effects, while co-culture showed FICi values of ≤ 0.5, suggesting a synergistic interaction. Treatment with gentamicin and fucoidan led to significant downregulation of the *icaA* and *bap* genes in both single-species and dual-species biofilms of *S. aureus* and *A. baumannii*. The reductions in gene expression were more pronounced in dual-species biofilms compared to single-species biofilms. Additionally, treatment with imipenem and fucoidan also resulted in significant downregulation of these genes in both biofilm types. Cytotoxicity assessments indicated that higher concentrations of fucoidan were toxic, yet no harmful effects were noted at the optimal synergistic concentrations used with antibiotics.

Conclusion In our investigation, we found that combining gentamicin, imipenem, and fucoidan had a synergistic effect on dual-species biofilms of *S. aureus* and *A. baumannii*, suggesting potential benefits for treating such infections effectively. This underscores the importance of understanding microbial interactions, antibiotic susceptibility, and biofilm formation in DFUs.

Keywords *Staphylococcus aureus*, *Acinetobacter baumannii*, Imipenem, Gentamicin, Fucoidan, Dual-species biofilms, Co-culture

Introduction

Diabetes mellitus is identified as a chronic metabolic disorder marked by ongoing hyperglycemia, stemming from the body's inability to produce or effectively utilize insulin [1]. The increasing global prevalence of diabetes presents considerable health, social, and economic challenges [2]. Among the various complications that arise from diabetes, diabetic foot ulcers (DFUs) stand out as particularly debilitating, often leading to severe infections, extended hospitalizations, and even amputations [3]. These chronic, non-healing wounds play a major role in the mortality rates among diabetic patients, emphasizing the critical necessity for effective management strategies [4].

Bacterial infections complicate DFUs and can often be polymicrobial [5]. *Staphylococcus aureus* and *Acinetobacter baumannii* are two of the most concerning pathogens associated with these ulcers [6]. Known for its ability to evade the immune response, *S. aureus*, especially its methicillin-resistant strains (MRSA), can cause a variety of infections [7]. *A. baumannii*, a highly adaptable Gram-negative bacterium, is infamous for its resistance to multiple antibiotics and its role in hospital-acquired infections [8]. The presence of these pathogens not only aggravates the severity of the wounds but also complicates treatment efforts due to their resilience.

Biofilm formation plays a crucial role in the persistence of these infections [9]. Biofilms consist of structured groups of bacteria that are enclosed in a self-produced extracellular matrix, allowing them to adhere to surfaces, including tissues and medical devices [10]. Within biofilms, bacteria demonstrate enhanced resistance to antibiotics and immune responses from the host [11]. The coexistence of *S. aureus* and *A. baumannii* within biofilms creates an even more significant challenge, as their interactions may bolster their survival mechanisms and resistance, complicating efforts to eradicate these infections [12].

Fucoidan, a type of sulfated polysaccharide, is abundantly produced by diverse algae species, and its biological properties have been extensively investigated [13]. The antimicrobial activity of fucoidan is attributed to its ability to hinder bacterial nutrient exchange and exert inhibitory effects on bacterial pathogens by modulating the bacterial cell wall permeability or forming a gel-like barrier on the bacterial membrane [14]. The escalating resistance of bacteria to conventional antibiotics has prompted the exploration of novel antimicrobial agents. Natural compounds, such as fucoidan, offer promise as potential sources of antimicrobial drugs due to their perceived lower side effects and cost-effectiveness.

Interactions among microbes that confer mutual benefits, particularly within mixed biofilms, play a significant role in increasing resistance to antimicrobial agents [15]. Combination therapy involves the simultaneous use of two or more active antibiotics [16]. This approach lowers the likelihood of drug resistance, reduces the need for high doses of potentially toxic drugs, and often produces more potent effects in biochemical activity compared to monotherapy [17, 18]. The majority of retrospective studies have indicated that combination therapy yields greater effectiveness compared to monotherapy [19–21]. The efficacy of combination therapy remains a subject of scientific debate. However, concurrent cultivation has shown a potential for heightened sensitivity in *S. aureus* and *P. aeruginosa*, prompting speculation

regarding similar behavior in *S. aureus* and *A. baumannii* [22, 23]. Research on the optimal antibiotic combinations for addressing infections caused by these isolates is currently limited. In this study, we explored the synergistic effects of combining gentamicin and imipenem antibiotics with fucoidan against both individual and co-culture planktonic conditions of *S. aureus* and *A. baumannii*, as well as in single-species biofilm and dual-species biofilm conditions.

Materials and methods

Ethical statement

Approval for the participation of human subjects was granted by the General Directorate of Health Affairs at Hamadan University of Medical Sciences, Iran, under ethics approval number IR.UMSHA.REC.1401.809. All clinical strains used in this study was obtained from routine medical testing or existing strain collections, ensuring that only bacterial cultures from these approved sources were utilized.

This research was conducted at Besat Hospital in Hamadan city and involved 48 patients diagnosed with type II diabetes mellitus, aged between 43 and 61 years, who were admitted with diabetic foot infections to the Internal Ward from January to April 2023. Additionally, consent was obtained from all participating patients before the commencement of the experiment. Swab samples (comprising aspirates and/or pus) were carefully gathered from heavily infected ulcers, employing sterile bottles and adhering to rigorous hygienic protocols.

Materials

Fucoidan, a sulfated polysaccharide, along with gentamicin and imipenem, was purchased from Sigma-Aldrich, USA. The fucoidan used in this study was extracted from the brown algae *Sargassum angustifolium*. Its structure is primarily composed of L-fucose and sulfate ester groups, with minor components of other monosaccharides such as galactose, mannose, xylose, and glucuronic acid.

Collection of clinical isolates of *S. aureus* and *A. baumannii*

A total of thirty-one isolates of *S. aureus* and twenty-seven isolates of *A. baumannii* were collected from clinical samples of DFU patients. These clinical samples were transferred to the microbiology laboratory at Hamadan University using transport media, including trypticase soy agar (TSA) and brain heart infusion (BHI).

Upon arrival at the laboratory, the initial identification of *S. aureus* was carried out using established protocols, followed by genetic confirmation through polymerase chain reaction (PCR), specifically targeting the amplification of the *nuc* gene [24]. A standard strain of *S. aureus* ATCC 25923 was employed as a positive control to ensure experimental consistency. Similarly, for *A. baumannii*, chemical identification was conducted using oxidase-catalase tests, motility tests, triple sugar iron (TSI) tests, and microscopic examination. Genetic confirmation of the *Acinetobacter* genus, as well as species determination, involved PCR amplification of the bla_{oxa51}-like gene, with *A. baumannii* ATCC 19606 serving as the positive control. The bla_{oxa51}-like gene products were subsequently sent for sequencing [25].

After the final identification, all *S. aureus* and *A. baumannii* isolates were preserved in trypticase soy broth (TSB) containing 20% glycerol at -70 °C for further analysis. All culture media and reagents used in this study were obtained from Merck, Germany.

Screening of *S. aureus* based on methicillin resistance

Isolates of *S. aureus* diagnosed for methicillin resistance were screened using the disk diffusion method with Cefoxitin (FOX; 30 µg) (MAST, UK) disk on Mueller-Hinton agar (MHA) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, employing standard zone size inhibition criteria. The confirmation was performed using the PCR method for the *mecA* gene [26]. *S. aureus* ATCC 33591 was used as a positive control, and *Escherichia coli* ATCC 25922 had served as a negative control in the PCR test.

Biofilm formation assay

The biofilm-forming ability of *S. aureus* and *A. baumannii* strains was assessed using the microtiter plate method, as previously described depending on the bacterial type [26]. To investigate dual-species biofilms formation, the biofilms of *S. aureus* were allowed to grow before the addition of *A. baumannii* to facilitate the survival of *S. aureus*. Colonies of *S. aureus* were inoculated in 5 mL TSB supplemented with 1% glucose (1% Glu TSB) and cultured overnight. Cultures were diluted to 5×10^5 CFU/mL in 1% Glu TSB, and 1.5 mL of the diluted *S. aureus* suspension was seeded onto 24-well plates (JET Biofil, China). The *S. aureus* biofilms were allowed to form for 24 h on a shaker (37 °C, 220 rpm). After washing and discarding planktonic cells, 1.5 mL of *A. baumannii* suspension with a concentration of 5×10^5 CFU/mL was added to the *S. aureus* biofilms. The dual-species biofilms were then incubated for an additional 18 h on a shaker (37 °C, 220 rpm). The wells were washed with 1.5 mL 1% Glu TSB, and planktonic cells from both species were removed. Adherent bacteria were fixed with 200 µL 99% methanol for 25 min. After removing the methanol, the wells were dried, and 200 µL of 2% crystal violet was added for 20 min to allow staining of biofilm-forming strains. The plates were emptied, and excess stain was washed off with sterile distilled water. Next, 200 µL of 33% glacial acetic acid was added to dissolve remaining colors completely. The plates were held at room

temperature for 15 min, and finally, the optical density (OD) of the wells was read at 570 nm wavelength using an ELISA reader (BioTek, Germany). The experiment was performed in triplicate for each bacterial sample, using a well containing 1% Glu TSB culture medium as a negative control. Biofilm formation was assessed by comparing the OD of each test well with that of the control well, and the results were classified into the following categories [27]:

Strong biofilm OD greater than four times the cut-off value (ODc).

Moderate biofilm OD between two and four times the ODc.

Weak biofilm OD between the ODc and two times the ODc.

Non-biofilm OD equal to or less than the ODc.

To examine the formation of dual-species biofilms prior to methanol addition, we cultured 10 µL of dual-species biofilms were cultured on selective Leeds media for *A. baumannii* and mannitol salt agar (MSA) media for *S. aureus* [8, 28]. This step occurred subsequent to the initial biofilm formation and before fixation with 99% methanol.

Antibiotic susceptibility pattern and MDR isolates

To establish the antibiotic sensitivity pattern, all isolates of *S. aureus* and *A. baumannii* were subjected to the Disk Diffusion method, following the CLSI recommendations for the year 2023 [29]. For *S. aureus*, the antibiotic susceptibility testing encompassed Gentamicin (GM; 10 µg), Clindamycin (CD; 2 µg), trimethoprim-sulfamethoxazole (TS; 1.25/23.75 µg), erythromycin (E; 15 µg), linezolid (LZD; 30 µg), imipenem (IMP; 10 µg), and vancomycin (VAN; 30 µg) (MAST, UK). Similarly, susceptibility testing for *A. baumannii* comprised imipenem (IMP; 10 µg), meropenem (MER; 10 µg), gentamicin (GM; 10 µg), Piperacillin (PIP; 100 µg), ampicillin (AMP; 10 µg), ceftriaxone (CEF; 30 µg), amoxicillin/clavulanic acid (AUG; 20/10 µg), tetracycline (TET; 30 µg), and ciprofloxacin (CIP; 5 µg) (MAST, UK). Subsequently, isolates that showed resistance to a minimum of three classes of different antibiotics were considered as multidrug-resistant (MDR) based on the defined criteria [30].

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and MBC/MIC

The broth microdilution technique, as per the CLSI guidelines, was employed to ascertain the MIC and MBC values for 10 strains of *S. aureus* and 10 strains of *A. baumannii* exhibiting resistance to two antibiotics, namely gentamicin and imipenem [29]. In brief, fresh bacterial isolates were cultured overnight in cation-adjusted Mueller-Hinton broth (caMHB) at 37 °C with shaking at 180 rpm. The following day, the bacterial cells were adjusted to the mid-logarithmic phase at a turbidity standard of 0.5 McFarland (equivalent to 10^8 CFUs/mL). The bacterial concentration was then adjusted to 10^6 CFUs/mL in the same medium. Simultaneously, two-fold serial dilutions of antibiotics and fucoidan were prepared in caMHB in a volume of 100 µL per well in a 96-well flat-bottom microplate (Jet Biofil, Guangzhou, China). The concentration ranges were as follows: gentamicin and imipenem (0.5 to 1024 µg/mL), and fucoidan (3.9 to 1000 µg/mL). Subsequently, 100 µL of the bacterial suspension containing 10^6 CFUs/mL was added to each well containing the serially diluted antibiotics or fucoidan. The microplates were incubated at 37 °C for 18–24 h. For co-culture contexts, 50 µL of each *S. aureus* and *A. baumannii* bacterial suspension, totaling 5×10^4 CFU/mL, were added in equal proportions (1:1) to the wells. Following incubation at 37 °C for 24 h, the MIC was determined as the lowest concentration of the antimicrobial agent capable of inhibiting bacterial growth. Additionally, to ascertain MBC, 10 µL from the last wells displaying no visible growth were streaked onto MHA plates and incubated at 37 °C for 24 h. MBC was defined as the minimum concentration of the antimicrobial substance capable of eradicating 100% of the initial bacterial inoculum (10^5 CFU). The process of determining MIC and MBC for antibiotics was repeated at least three times for accuracy and consistency.

Determination of minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC)

The MBIC and MBEC were evaluated for the aforementioned 20 isolates. In summary, fresh bacterial colonies were cultivated overnight in 5 mL of 1% Glu TSB at 37 °C with shaking at 180 rpm. The bacterial cells were first adjusted to a turbidity standard of 0.5 McFarland (equivalent to 10^8 CFU/mL). The concentration was then further diluted to 10^6 CFU/mL in the same medium. Subsequently, an inoculum of 10^6 CFUs was added to the wells of U-shaped microplates filled with 1% Glu TSB medium. The method mentioned was used in dual-species biofilm cases. The plates were incubated for 24 h at 37 °C. Wells containing bacteria grown in 1% Glu TSB without exposure to antimicrobial agents were considered positive controls, while wells containing 1% Glu TSB without bacteria served as negative controls. MBIC was calculated as the lowest concentration of the antibiotic that caused 90% inhibition of biofilm formation in the tested isolate according to the formula [31]:

$$\mathrm{MBIC} = [1 - (\mathrm{OD\,test} / \mathrm{OD\,control})] \times 100\%$$

To assess the eradication effects MBEC, biofilms were first formed using the above method. Subsequently, the wells were washed three times with PBS solution (200 µL) and air-dried. Serial dilutions of gentamicin, imipenem and fucoidan were added to the wells containing the formed biofilm and incubated for 24 h at 37 °C. In the next step, after discarding the liquid, the wells were washed three times with PBS, and after scraping the well contents and mixing with physiological saline, 10 µL of the well contents were cultured on MHA medium and incubated at 37 °C for 48 h. MBEC of gentamicin and imipenem was considered as the minimum amount of antimicrobial agent required to eliminate 100% of bacteria [32]. The positive control for this test consisted of standard isolates forming biofilm in 1% Glu TSB without exposure to antimicrobial agents, and the negative control was 1% Glu TSB without bacteria.

Determination of the synergistic effects in the planktonic state of isolates

The synergistic effects of gentamicin, imipenem and fucoidan were evaluated for six selected isolates, including *S. aureus* 6, *S. aureus* 7, *S. aureus* 22, *A. baumannii* 1, *A. baumannii* 8, and *A. baumannii* 20, utilizing the checkerboard microdilution method. These approaches were applied in individual culture studies, drawing from established methodologies [33]. Briefly, serial dilutions of gentamicin (0.25 to 1024 µg/mL), imipenem (0.25 to 1024 µg/mL), and fucoidan (3.9 to 1000 µg/mL) were prepared in 100 µL volumes per well within 96-well plates. Columns 1 to 11 of the plates, each containing 2-fold serial dilutions of gentamicin, were paired with rows A to G, housing 2-fold serial dilutions of imipenem. Column 12 featured serial dilutions of gentamicin alone, while row H contained serial dilutions of imipenem. Following the generation of 0.5 McFarland turbidity and dilution, 100 µL equivalent to 10^5 CFU/mL of bacterial suspension was added to the wells. In the co-cultivation experiments performed for the first time in our study, 50 µL (equivalent to 5×10^4 CFU/mL) of both *S. aureus* and *A. baumannii* bacteria were concurrently introduced in equal proportions (1:1) to the wells.

The fractional inhibitory concentration index (FICi) for the combined antibacterial agents was calculated as follows [34]:

$$\begin{aligned}\mathrm{FICiA/B} = {} & (\mathrm{MIC\ drug\ A\ in\ combination/MIC\ drug\ A\ alone}) \\ & + (\mathrm{MIC\ drug\ B\ in\ combination/MIC\ drug\ B\ alone})\end{aligned}$$

The obtained value was interpreted as follows: less than 0.5 indicated synergy, between 0.5 and 1 indicated relative synergy, equal to 1 indicated an additive effect, between 1 and 4 indicated no interaction, and more than 4 indicated antagonism [35].

Moreover, to explore the interactions among antibacterial drugs, the MBC values were assessed using the broth microdilution checkerboard technique, referred to as the fractional bactericidal concentration index (FBCi). This method for evaluating antibacterial interactions was similar to the FIC method. A 100 µL aliquot of diluted bacterial stock at 10^5 CFUs/mL was added to each well, followed by incubation at 37 °C for 24 h. After incubation, 10 µL from each well was plated on MHA, allowing for the determination of the MBC values for gentamicin, imipenem, and fucoidan, defined as the lowest concentrations needed to eliminate 100% of the cultured isolates. The FBCi was then calculated using the formula:

$$\begin{aligned}\mathrm{FBCiA/B} = {} & (\mathrm{MBC\ of\ drug\ A\ in\ combination/MBC\ of\ drug\ A\ alone}) \\ & + (\mathrm{MBC\ of\ drug\ B\ in\ combination/MBC\ of\ drug\ B\ alone})\end{aligned}$$

These FBCi indices were employed to identify the type of interaction between the antibacterial agents, analogous to the FICi method [36].

Determining the synergistic effects in the biofilm state of isolates

Utilizing MBIC, the fractional biofilm inhibitory concentration index (FBICi) for six isolates, including *S. aureus* 6, *S. aureus* 7, *S. aureus* 22, *A. baumannii* 1, *A. baumannii* 8, and *A. baumannii* 20, was determined. Briefly, preformed dual-species biofilms were established in 96 U-shape microplates over 24 h. Subsequently, serial dilutions of antibiotics ranging from 1 to 1024 µg/mL (100 µL each) and fucoidan (3.9 to 1000 µg/mL) were added to the wells, followed by overnight incubation at 37 °C. The FBICi for combined agents was calculated using the formula [36]:

$$\begin{aligned}\mathrm{FBICi_{A/B}} = {} & (\mathrm{MBIC\ drug\ A\ in\ combination/\ MBIC\ drug\ A\ alone}) \\ & + (\mathrm{MBIC\ drug\ B\ in\ combination/\ MBIC\ drug\ B\ alone})\end{aligned}$$

The resultant values indicated the nature of drug interaction as described above.

Additionally, employing MBEC, a novel formula, termed Fractional biofilm eradication concentration index (FBECi), was introduced to assess the interaction between antimicrobial agents against the biofilm form. FBECi for two combined anti-biofilm agents was calculated using the formula:

$$\begin{aligned}\mathrm{FBECi_{A/B}} = {} & (\mathrm{MBEC\ drug\ A\ in\ combination/\ MBEC\ drug\ A\ alone}) \\ & + (\mathrm{MBEC\ drug\ B\ in\ combination/\ MBEC\ drug\ B\ alone})\end{aligned}$$

The resulting values conveyed the type of drug interaction akin to the FBICi calculations mentioned earlier.

Field emission scanning electron microscopy (FE-SEM)

Field emission scanning electron microscopy (FE-SEM) was employed to visualize the synergistic effects of gentamicin, imipenem, and fucoidan at their FBIC amounts on dual-species biofilms of *S. aureus* and *A. baumannii*. Two strains were used in this study: *S. aureus* 6 and *A. baumannii* 1. Sample preparation was conducted according to the protocol described by Shams Khozani et al., with some modifications [37]. Briefly, fresh bacterial cultures were grown in 1% Glu TSB at 37 °C for 24 h. The biofilms were then treated with combinations of gentamicin-imipenem, gentamicin-fucoidan, and imipenem-fucoidan, each incubated with a bacterial suspension at a concentration of 1.5×10^7 CFU/mL at 37 °C for 24 h, according to their respective FBIC doses. Prior to incubation, sterile slides were cut and placed into the wells. Following incubation, the slides were gently washed three times with sterile distilled water and then fixed in 2.5% glutaraldehyde in 1x PBS for 3 h at room temperature. The slides were subsequently rinsed three times in distilled water and post-fixed in 1.5% osmium tetroxide for 1 h. Following three additional rinses in distilled water, the samples were dehydrated using graded alcohol concentrations of 20%, 30%, 50%, 70%, 80%, 90%, and 100%, with each step lasting 10 min. The specimens were then mounted on conductive copper SEM tape, coated with gold nanoparticles, and examined using an FE-SEM instrument (MIRA3, TESCAN Co., Czechia).

Effect of antimicrobials on the biofilm encoding genes

Biofilm-forming strains were chosen for real-time PCR analysis to examine the expression of biofilm-related genes *icaA* in *S. aureus* and *bap* in *A. baumannii*. After creating single-species biofilm and dual-species biofilms, these strains were treated overnight with 1/2 FBIC concentrations of synergistic gentamicin, imipenem and fucoidan. The following day, total RNA was extracted using a RNX-plus Mini Kit (Sinaclon, Iran), according to the manufacturer's guidelines. The RNA concentration, purity, and integrity were evaluated. Subsequently, 1 µg of RNA was used for a cDNA Synthesis kit (Parstous Biotechnology, Iran), following the manufacturer's instructions. Gene expression was then assessed using the 2X Q-PCR Master Mix with 2 µL of cDNA and 1 µL of each *icaA*, *bap*, and 16 S rRNA primers in a 20 µL volume on a real-time PCR device (LightCycler® 96 Instrument, Roche, United States). The primers for *icaA*, *bap*, and 16 S rRNA were obtained from previous studies [38, 39]. A standard curve, created using serial dilutions of mRNA from untreated *S. aureus*ATCC 29213 for *icaA* and untreated *A. baumannii* ATCC 19606 for *bap*, was used to ensure amplification efficiency [40]. Gene expression was ultimately calculated using the Ct assay, with 16 S rRNA genes serving as internal controls for each bacterium.

Toxicity assays

The cytotoxicity of fucoidan on host cells was assessed using the MTT assay [41]. Human Skin Fibroblast cells (HSF-PI 16) were cultivated in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). The cells were maintained at 37 °C with 5% CO_2 and 95% relative humidity until they reached a density of 4×10^4 cells per well and were then cultured overnight. Subsequently, various concentrations of fucoidan, ranging from 1000 to 3.9 µg, were prepared. Additionally, combinations of fucoidan at doses of 125 µg and 62.5 µg with imipenem at concentrations of 32 µg and 64 µg, as well as fucoidan at 125 µg and 62.5 µg with gentamicin at 32 µg and 64 µg, were added to a 96-well microplate. The plates were then incubated for 24 h at 37 °C. The supernatant was aspirated, and 100 µL of DMSO was included in each well to dissolve the formazan crystals. The absorbance at 570 nm was determined using a spectrophotometer (BioTek, USA). Cell viability was computed using the formula: Percentage survival = (OD test / OD control) × 100.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, United States). A t-test was used to evaluate the significance of the findings regarding the anti-biofilm effect of the combination of antibiotics. Additionally, the ANOVA test was employed to compare the survival rates of the HSF-PI 16 cell line exposed to different concentrations of fucoidan relative to the control group and the gene expression between the single-species biofilm isolates and the dual-species biofilms, as well as the FBIC values. All assays were conducted with a confidence level of 95%, and a p-value of less than 0.05 was considered statistically significant. To describe the correlation between the examined concentrations and the percentage of activities, a non-linear regression test was performed. All experiments were conducted in triplicate.

Result

MRSA and biofilm production analysis

The findings from both antibacterial sensitivity assessments and molecular analyses, focusing on the FOX disc and the *mecA* gene, revealed that 80.6% ($n=25$) of *S. aureus* isolates exhibited characteristics consistent with Methicillin-Resistant *S. aureus* (MRSA). Notably, a considerable proportion of both *S. aureus* and *A. baumannii* isolates demonstrated variable levels of biofilm formation, as evidenced by OD values ranging from 0.2 to 2.8 across all isolates. Furthermore, based on these findings,

the isolates' biofilm-forming capacity was classified into strong, intermediate, and weak producers, as detailed in both Tables 1 and 2.

Antibiotic susceptibility patterns and MDR isolates

An examination of disk diffusion data from clinical isolates was undertaken to evaluate antibiotic resistance rates. In *S. aureus*, resistance rates to IMP, TS, E, CD, GM, LZD, and VAN were determined as 80.6%, 67.7%, 64.5%, 58.5%, 51.6%, 0%, and 0%, respectively. Similarly, antibiotic resistance rates for selected clinical isolates of *A. baumannii*, based on disk diffusion data, were as follows: PIP 100%, CEF 100%, AM 100%, TET 92.6%, AUG 85.1%, GM 77.7%, MER 77.7%, CIP 74.0%, and IMP 55.5%. Overall, 77.4% of *S. aureus* isolates and 100% of *A. baumannii* isolates exhibited MDR. Further information regarding the antimicrobial susceptibility testing of antibiotics against the isolates is provided in Tables 1 and 2. The statistical analysis is essential in assessing the relationship between MDR and biofilm formation ($P<0.05$). Our findings reveal a noteworthy association between antibiotic resistance and the presence of strong biofilm formations among *S. aureus* clinical isolates ($P<0.05$). Refer to Table 3 for further details.

Ability to formation of dual-species biofilms

We investigated various conditions for all isolates (31 *S.aureus* and 27 *A. baumanii*), revealing that 72 conditions formed strong dual-species biofilms, while 67 formed moderate dual-species biofilms out of a total 837 conditions. Detailed information for all isolates is presented in Table 4; Fig. 1. In some cases, despite the fact that OD was higher than the negative control, the growth of both bacteria after biofilm formation was not observed in culture on MSA and Leeds media, in which case it was reported as non-biofilm formation.

Table 1 Characteristics of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, multiple drug-resistance (MDR) isolates, antibiotic resistance patterns, and biofilm formation types in *Staphylococcus aureus*

Strain	MRSA	FOX	GM	CD	TS	E	LZD	IMP	VAN	MDR/ Non-MDR	Biofilm
S. aureus 1	1	R	R	R	R	R	S	R	S	MDR	Strong
S. aureus 2	0	S	S	S	S	S	S	R	S	Non-MDR	Moderate
S. aureus 3	1	R	R	S	R	R	S	R	S	MDR	Strong
S. aureus 4	1	R	R	S	R	S	S	S	S	MDR	Weak
S. aureus 5	1	R	R	R	R	S	S	R	S	MDR	Moderate
S. aureus 6	1	R	R	R	S	R	S	R	S	MDR	Strong
S. aureus 7	1	R	R	R	R	R	S	R	S	MDR	Strong
S. aureus 8	1	R	S	S	S	S	S	R	S	Non-MDR	Moderate
S. aureus 9	1	R	S	S	S	S	S	R	S	Non-MDR	Moderate
S. aureus 10	1	R	S	R	R	R	S	R	S	MDR	Weak
S. aureus 11	1	R	R	S	R	R	S	S	S	MDR	Strong
S. aureus 12	1	R	R	R	R	S	S	R	S	MDR	Moderate
S. aureus 13	1	R	S	R	S	R	S	R	S	MDR	Strong
S. aureus 14	1	R	R	S	R	R	S	R	S	MDR	Moderate
S. aureus 15	1	R	S	S	S	S	S	S	S	Non-MDR	Moderate
S. aureus 16	1	R	R	R	R	R	S	R	S	MDR	Strong
S. aureus 17	0	S	S	S	R	S	S	R	S	Non-MDR	Weak
S. aureus 18	1	R	S	R	S	R	S	R	S	MDR	Moderate
S. aureus 19	0	S	S	R	S	R	S	R	S	MDR	Moderate
S. aureus 20	1	R	R	R	R	R	S	R	S	MDR	Strong
S. aureus 21	0	S	S	R	S	R	S	S	S	Non-MDR	Moderate
S. aureus 22	0	S	R	R	R	R	S	R	S	MDR	Strong
S. aureus 23	1	R	S	S	R	R	S	R	S	MDR	Weak
S. aureus 24	1	R	R	R	R	R	S	R	S	MDR	Moderate
S. aureus 25	1	R	R	S	R	S	S	R	S	MDR	Weak
S. aureus 26	1	R	S	R	R	R	S	R	S	MDR	Moderate
S. aureus 27	1	R	R	R	R	S	S	S	S	MDR	Strong
S. aureus 28	1	R	R	R	R	R	S	R	S	MDR	Strong
S. aureus 29	1	R	S	S	R	R	S	S	S	MDR	Weak
S. aureus 30	1	R	S	R	R	R	S	R	S	MDR	Strong
S. aureus 31	0	S	S	S	S	S	S	R	S	Non-MDR	Weak

Abbreviation: MRSA, Methicillin-resistant *Staphylococcus aureus*; FOX, cefoxitin; GM, gentamicin; CD, clindamycin; TS, trimethoprim-sulfamethoxazole; E, erythromycin; LZD, linezolid; IMP, imipenem; VAN, vancomycin; R, resistant; I, intermediate; S, sensitive; MDR, multiple drug-resistance

Table 2 Characteristics of multiple drug-resistance (MDR) isolates, antibiotic resistance patterns, and biofilm formation types in *Acinetobacter baumannii*

Strain	IMP	MER	GM	PIP	AUG	CEF	AM	TET	CIP	MDR/ Non-MDR	Biofilm
A. baumannii 1	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 2	R	R	R	R	R	R	R	R	S	MDR	Weak
A. baumannii 3	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 4	R	R	R	R	R	R	R	R	S	MDR	Strong
A. baumannii 5	S	S	R	R	R	R	R	R	S	MDR	Strong
A. baumannii 6	S	R	R	R	S	R	R	S	S	MDR	Moderate
A. baumannii 7	I	R	R	R	R	R	R	R	R	MDR	Weak
A. baumannii 8	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 9	S	S	R	R	I	R	R	R	R	MDR	Strong
A. baumannii 10	S	R	S	R	R	R	R	R	R	MDR	Strong
A. baumannii 11	R	R	S	R	R	R	R	I	R	MDR	Moderate
A. baumannii 12	S	S	S	R	R	R	R	R	R	MDR	Moderate
A. baumannii 13	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 14	S	S	S	R	R	R	R	R	R	MDR	Strong
A. baumannii 15	S	R	S	R	R	R	R	R	R	MDR	Moderate
A. baumannii 16	R	R	R	R	R	R	R	R	S	MDR	Moderate
A. baumannii 17	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 18	S	R	R	R	R	R	R	R	R	MDR	Moderate
A. baumannii 19	S	S	R	R	S	R	R	R	R	MDR	Strong
A. baumannii 20	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 21	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 22	R	R	R	R	R	R	R	R	R	MDR	Moderate
A. baumannii 23	S	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 24	S	I	S	R	S	R	R	R	S	MDR	Weak
A. baumannii 25	R	R	R	R	R	R	R	R	R	MDR	Moderate
A. baumannii 26	R	R	R	R	R	R	R	R	R	MDR	Strong
A. baumannii 27	R	R	R	R	R	R	R	R	S	MDR	Moderate

Abbreviation: IMP, imipenem; MER, meropenem; GM, gentamicin; PIP, piperacillin; AMP, ampicillin; CEF, ceftriaxone; AUG, amoxicillin/clavulanic acid; TET, tetracycline; CIP, ciprofloxacin; R, resistant; I, intermediate; S, sensitive; MDR, multiple drug-resistance

Table 3 Comparison of biofilm phenotypic patterns between MDR and non-MDR *Staphylococcus aureus* clinical isolates

Biofilm Mode	MDR; No (%) (*n*=24)	Non-MDR; No (%) (*n*=7)	Total; No (%) (*n*=31)	*P*-value
Strong	12 (50.0)	0 (0)	12 (38.7)	0.017
Moderate	9 (37.5)	3 (42.8)	12 (38.7)	0.798
Weak	3 (12.5)	4 (57.2)	7 (22.5)	0.145

Abbreviation: MDR, multiple drug-resistance

MIC and MBC ranges and MBC/MIC values

In isolated individual cultures of *S. aureus*, the geometric mean MIC values were 12.12 µg/mL for gentamicin, 19.84 µg/mL for imipenem, and 48.50 µg/mL for fucoidan. For isolated individual cultures *A. baumannii*, these values were 22.62 µg/mL for gentamicin, 9.18 µg/mL for imipenem, and 31.20 µg/mL for fucoidan. Similarly, the geometric mean MBC values for gentamicin, imipenem and fucoidan in *S. aureus* were 119.42, 48.50, and 88.37 µg/mL, respectively, while for *A. baumannii*, they were 128, 14.92 and 62.62 µg/mL, respectively. Additionally, the geometric mean values for MBC/MIC for gentamicin, imipenem and fucoidan were 9.84, 2.46, and 2.14 respectively, in *S. aureus*, and 5.65, 1.74, and 2 respectively, in *A. baumannii*. For further details, refer to Table 5.

In the co-culture condition, we selected six isolates for investigation: *S. aureus* 6, *S. aureus* 7, *S. aureus* 22, *A. baumannii* 1, *A. baumannii* 8, and *A. baumannii* 20. These isolates are capable of forming dual-species biofilms under nine different conditions (3×3). Among these, eight conditions lead to strong dual-species biofilms, while one condition results in a moderate dual-species biofilm. MIC and MBC values were assessed in co-culture contexts where inhibition or eradication of both bacterial species occurred, as detailed in Table 5; Fig. 2A. The geometric mean MIC and MBC values, as well as the MBC/MIC ratios for gentamicin, imipenem and fucoidan, were calculated as follows: 5.87 µg/mL, 11.75 µg/mL, and 1.85 for gentamicin, 3.70 µg/mL, 8 µg/mL, and 2.16 for imipenem, and 19.65 µg/mL, 42.48 µg/mL, and 2.16 for fucoidan respectively.

Table 4 Data regarding the formation of dual-species biofilm

Strain	S.a.1	S.a.2	S.a.3	S.a.4	S.a.5	S.a.6	S.a.7	S.a.8	S.a.9	S.a.10	S.a.11	S.a.12	S.a.13	S.a.14	S.a.15	S.a.16	S.a.17	S.a.18	S.a.19	S.a.20	S.a.21	S.a.22	S.a.23	S.a.24	S.a.25	S.a.26	S.a.27	S.a.28	S.a.29	S.a.30	S.a.31
A.b.1	M	N	S	N	W	S	S	M	M	N	M	W	M	W	W	W	W	W	W	M	W	S	W	M	W	N	S	S	W	S	N
A.b.2	W	N	W	N	W	W	W	W	W	N	W	N	W	W	W	W	W	W	W	W	W	W	W	W	W	N	W	W	W	W	N
A.b.3	S	N	W	N	W	S	S	M	W	N	M	W	W	N	W	W	N	W	W	W	W	W	W	M	W	N	S	S	W	S	N
A.b.4	W	N	W	N	W	W	W	W	W	N	W	N	W	N	W	W	N	W	W	W	W	W	W	W	W	N	W	W	W	W	N
A.b.5	M	N	W	N	W	M	M	M	W	N	W	N	W	W	W	W	N	W	W	W	W	W	N	W	W	N	W	W	W	W	N
A.b.6	W	N	W	N	W	W	W	W	W	N	W	N	W	W	W	W	W	N	W	W	W	W	N	W	W	N	W	W	W	W	N
A.b.7	W	N	W	N	N	W	W	W	W	N	W	N	W	W	W	W	W	N	W	W	W	W	N	W	W	N	W	W	W	W	N
A.b.8	S	N	S	N	N	S	S	M	W	N	S	N	S	W	W	S	W	N	W	S	W	S	W	M	N	N	S	M	W	W	N
A.b.9	M	N	W	N	N	S	S	W	W	N	W	M	W	W	N	S	N	N	W	S	W	S	W	W	N	N	W	W	W	W	N
A.b.10	W	N	W	N	N	W	W	W	W	N	W	W	W	W	N	W	N	N	W	W	N	W	W	W	N	N	W	W	N	W	N
A.b.11	M	N	S	N	N	S	S	M	M	N	M	W	M	W	N	W	N	N	N	M	N	W	W	M	N	N	S	S	N	S	N
A.b.12	W	N	W	N	W	W	W	W	W	N	W	M	W	W	W	W	N	N	N	W	N	W	W	W	N	N	W	W	N	W	N
A.b.13	S	N	W	N	W	S	S	M	W	N	M	W	W	W	W	W	N	N	N	W	N	S	W	M	N	N	S	S	N	S	N
A.b.14	W	N	W	N	W	W	W	W	N	N	W	W	W	W	W	W	W	N	N	W	N	W	W	W	N	N	W	W	W	W	N
A.b.15	N	N	W	N	W	M	W	W	N	N	W	W	S	M	S	M	M	W	W	M	N	W	W	W	N	N	W	N	W	W	N
A.b.16	N	N	W	N	W	M	W	W	W	N	W	M	W	W	M	W	W	W	W	W	W	W	W	W	N	N	W	N	W	W	N
A.b.17	N	N	W	N	W	M	S	W	W	N	W	M	M	W	W	W	W	W	W	W	W	S	W	M	W	N	W	N	W	W	N
A.b.18	W	N	M	N	N	W	S	W	W	N	W	M	S	M	N	W	W	N	W	N	W	W	W	S	W	N	W	N	W	W	N
A.b.19	M	N	S	N	N	S	S	W	M	N	M	W	M	W	N	W	W	N	W	N	W	W	N	M	W	N	S	M	W	W	N
A.b.20	W	N	W	N	W	S	M	W	W	N	W	W	W	W	N	W	W	N	W	N	W	S	N	W	W	N	W	W	W	W	N
A.b.21	S	N	W	N	W	W	S	M	W	N	M	W	W	W	W	W	N	W	W	N	W	S	W	M	W	N	W	W	W	M	N
A.b.22	W	N	W	N	W	W	W	W	W	N	W	W	W	W	W	W	N	W	W	W	W	W	W	W	W	N	S	S	W	S	N
A.b.23	N	N	N	N	N	N	W	W	N	N	M	W	S	M	S	M	M	S	M	M	M	M	W	S	W	N	W	W	W	W	N
A.b.24	W	N	W	N	W	W	W	W	N	N	W	W	W	W	M	W	W	W	W	W	W	W	W	W	W	N	S	S	W	S	N
A.b.25	W	N	W	N	W	W	S	W	N	N	W	W	W	S	W	W	W	W	W	W	W	S	W	W	W	N	W	W	W	W	N
A.b.26	W	N	W	N	W	W	S	W	W	N	W	W	M	M	W	N	N	W	W	W	W	S	W	W	W	N	W	W	W	W	N
A.b.27	W	N	W	N	W	W	M	W	W	N	N	N	N	W	W	W	W	W	W	W	W	W	W	W	N	N	N	N	W	W	N

Abbreviation: A.b, *A. baumannii*; S.a, *S. aureus*; S, strong; M, moderate; W, weak; N, non-biofilm

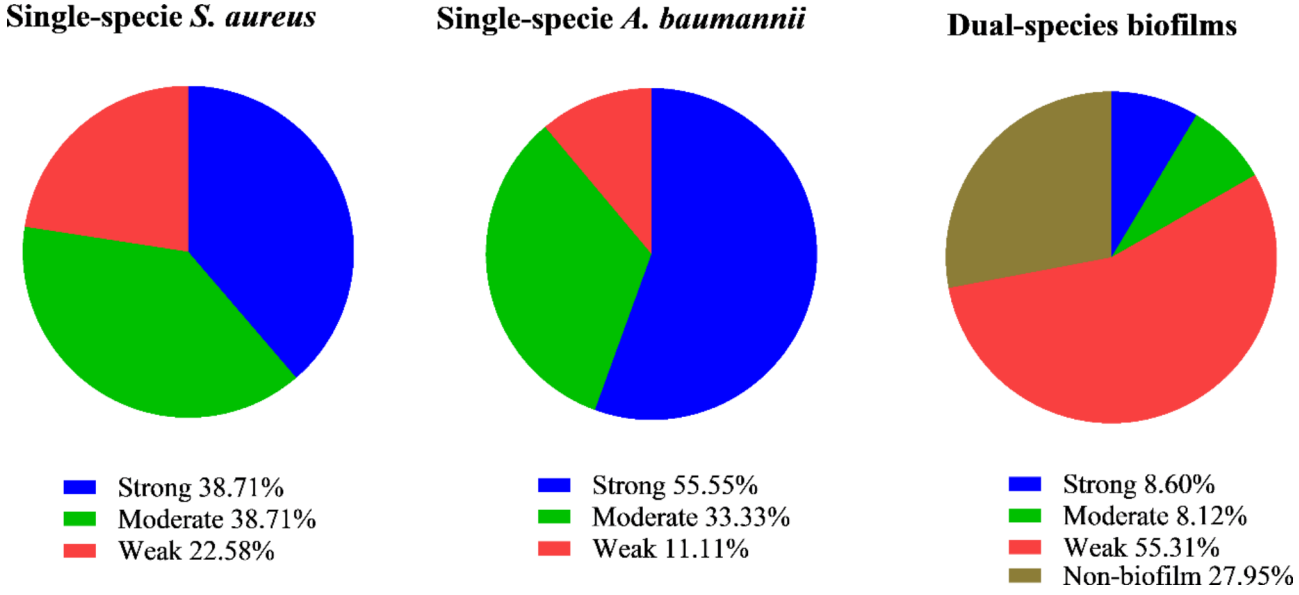

Fig. 1 Biofilm formation classification based on optical density (OD) values in single-species and dual-species biofilms of Staphylococcus aureus and Acinetobacter baumannii

Table 5 Ranges of MIC, MBC, and MBC/MIC ratio for gentamicin, imipenem and fucoidan

Strain	GM (μg/mL)			IMP (μg/mL)			FUC (μg/mL)		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
S. aureus 3	64	512	8	64	256	4	62.5	125	2
S. aureus 6	4	32	8	32	64	2	31.2	62.5	2
S. aureus 7	32	128	4	16	32	2	62.5	125	2
S. aureus 10	32	256	8	16	32	2	62.5	125	2
S. aureus 13	16	256	16	16	64	4	31.2	125	4
S. aureus 16	4	128	32	64	128	2	62.5	125	2
S. aureus 20	4	64	16	8	16	2	15.6	31.2	2
S. aureus 22	16	128	8	64	256	4	31.2	62.5	2
S. aureus 28	8	64	8	8	16	2	31.2	62.5	2
S. aureus 30	8	64	8	4	8	2	62.5	125	2
A. baumannii 1	64	128	2	32	64	2	125	256	2
A. baumannii 3	16	128	4	8	16	2	31.2	62.5	2
A. baumannii 4	32	128	8	32	64	2	15.6	62.5	4
A. baumannii 8	8	128	16	4	8	4	31.2	62.5	2
A. baumannii 13	64	512	8	8	8	1	15.6	31.2	2
A. baumannii 17	8	128	16	2	4	2	31.2	62.5	2
A. baumannii 20	64	256	4	16	16	1	15.6	31.2	2
A. baumannii 22	32	128	4	16	16	1	62.5	62.5	1
A. baumannii 25	32	64	2	4	8	2	31.2	62.5	2
A. baumannii 26	4	32	8	8	16	2	31.2	62.5	2
S. aureus 6* *A. baumannii* 1	8	16	2	2	4	2	15.6	31.2	2
S. aureus 7* *A. baumannii* 1	16	32	2	8	16	2	31.2	62.5	2
S. aureus 22* *A. baumannii* 1	8	8	1	4	8	2	15.6	31.2	2
S. aureus 6* *A. baumannii* 8	16	128	4	8	16	2	15.6	31.2	2
S. aureus 7* *A. baumannii* 8	4	4	1	8	16	2	31.2	62.5	2
S. aureus 22* *A. baumannii* 8	8	16	2	2	4	2	15.6	62.5	4
S. aureus 6* *A. baumannii* 20	8	16	2	4	8	2	31.2	62.5	2
S. aureus 7* *A. baumannii* 20	2	4	2	2	8	4	15.6	31.2	2
S. aureus 22* *A. baumannii* 20	1	2	2	2	4	2	15.6	31.2	2

Abbreviation: IMP, imipenem; GM, gentamicin; FUC, fucoidan, MIC, minimum inhibitory concentrations; MBC, minimum bactericidal concentrations

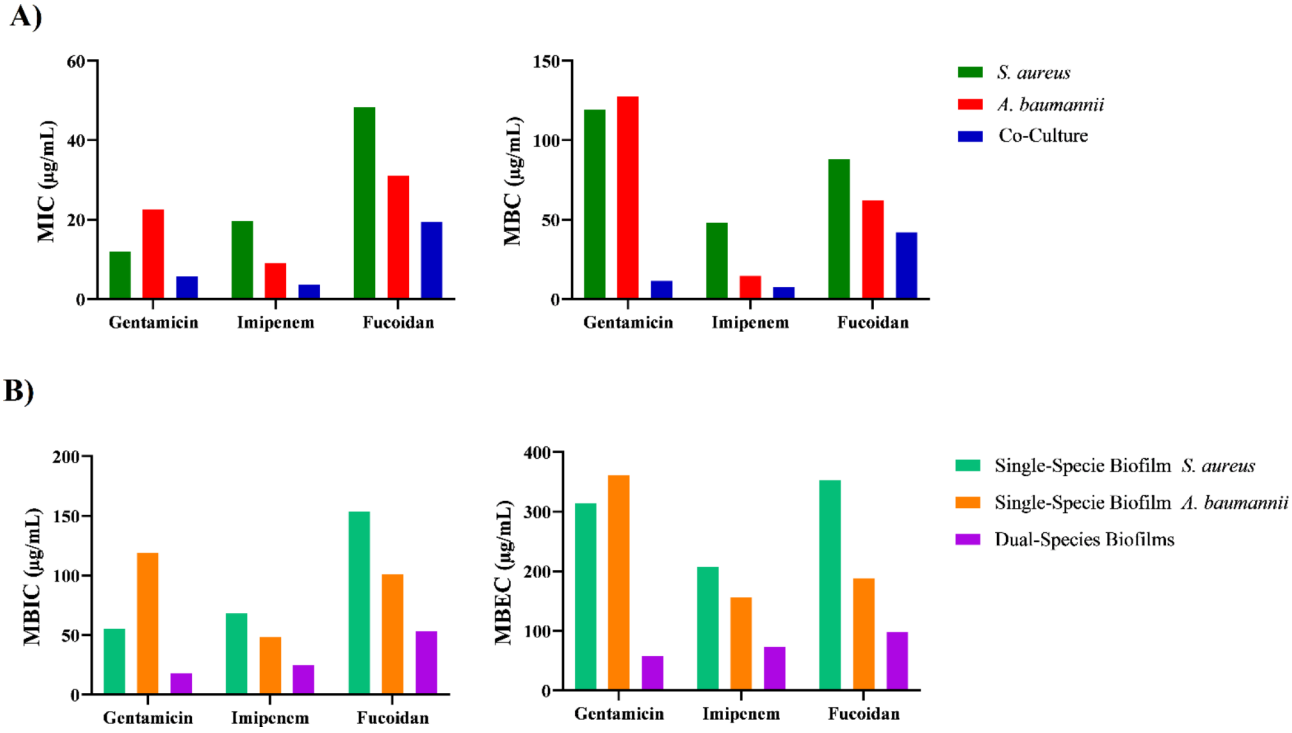

Fig. 2 **A**) The geometric mean values of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for both *Staphylococcus aureus* and *Acinetobacter baumannii*, under planktonic conditions. These values are shown for each species individually, as well as when the two bacteria are co-cultured. **B**) The geometric mean values of the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) are depicted for *S. aureus* and *A. baumannii* in both single-species biofilms and in dual-species biofilms, where both bacteria are present together

Evaluation of synergy in the planktonic state

In the present study, the geometric mean values for the most effective synergistic concentrations of gentamicin and imipenem, as determined by FICi, against isolates of *S. aureus* and *A. baumannii* across co-culture conditions of three selected isolates were 0.46, 0.34, and 0.21, respectively. For the combination of gentamicin and fucoidan, the geometric mean values were 0.63, 0.50, and 0.22, respectively. Additionally, for imipenem and fucoidan, the values were 0.71, 0.47, and 0.32, respectively.

Furthermore, the geometric mean values for the optimal synergistic concentrations, assessed by FBCi, revealed that for gentamicin and imipenem against *S. aureus* and *A. baumannii* isolates, the values were 0.42, 0.30, and 0.27, respectively. For gentamicin and fucoidan, the geometric mean values were 0.66, 0.59, and 0.22, respectively. Lastly, for imipenem and fucoidan, the values were 0.43, 0.45, and 0.33, respectively. Detailed information is provided in Table 6.

MBIC and MBEC ranges

In isolated samples of *S. aureus*, the geometric mean MBIC values were 55.71 µg/mL for gentamicin, 68.59 µg/mL for imipenem, and 153.89 µg/mL for fucoidan. For isolated *A. baumannii* samples, the corresponding values were 119.42 µg/mL for gentamicin, 48.50 µg/mL for imipenem, and 101.49 µg/mL for fucoidan.

Additionally, the geometric mean MBEC values for *S. aureus* were 315.17 µg/ml for gentamicin, 207.93 µg/mL for imipenem, and 353.55 µg/mL for fucoidan. For *A. baumannii*, the values were 362.03 µg/mL for gentamicin, 157.58 µg/mL for imipenem, and 189.46 µg/mL for fucoidan.

In dual-species biofilms, the geometric mean MBIC and MBEC values were 18.66 µg/mL and 59.25 µg/mL for gentamicin, 25.39 µg/mL and 74.65 µg/mL for imipenem, and 53.53 µg/mL and 99.19 µg/mL for fucoidan, respectively. These results are further detailed in Table 7; Fig. 2B.

Evaluation of synergy in the Biofilm condition

In our investigation, we determined the geometric mean values for the most potent synergistic concentrations of various antibiotic combinations against isolates of *S. aureus* and *A. baumannii* under dual-species biofilm conditions. Using the FBICi, we found that for gentamicin and imipenem, the values were 0.82, 0.59, and 0.29 across three selected isolates. For gentamicin and fucoidan, the values were 0.47, 0.44, and 0.28, respectively.

Table 6 The optimal synergistic concentrations of gentamicin-imipenem, gentamicin-fucoidan, and imipenem-fucoidan against planktonic cultures

Strain	FIC-indices (GEN+IMP)	FBC-indices (GEN+IMP)	FIC-indices (GEN+FUC)	FBC-indices (GEN+FUC)	FIC-indices (IMP +FUC)	FBC-indices (IMP +FUC)
S. aureus 3	1.25	0.75	0.25	0.25	0.5	0.75
S. aureus 6	0.25	0.25	0.75	0.75	0.375	0.187
S. aureus 7	0.25	0.375	1	1.25	1.25	1
S. aureus 10	0.25	0.125	0.375	0.5	0.25	0.093
S. aureus 13	1.25	1	1.25	1	1.25	1
S. aureus 16	0.375	0.375	1	1.25	1	1.25
S. aureus 20	0.375	0.25	0.5	0.25	0.5	0.375
S. aureus 22	0.75	1	0.25	0.75	1.25	0.187
S. aureus 28	1	1.25	1.25	1.25	0.75	1.25
S. aureus 30	0.187	0.187	0.75	0.5	1	0.187
A. baumannii 1	0.75	0.25	0.375	0.25	0.75	0.375
A. baumannii 3	0.5	0.25	1	1.25	0.75	1.25
A. baumannii 4	0.25	0.375	0.375	0.5	0.25	0.5
A. baumannii 8	0.5	0.187	0.5	0.375	0.75	0.187
A. baumannii 13	0.187	0.187	0.25	0.75	0.375	0.5
A. baumannii 17	0.75	0.5	1.25	1	1.25	1.25
A. baumannii 20	0.25	0.375	1	1.25	0.5	0.75
A. baumannii 22	0.187	0.5	0.25	0.75	0.25	0.187
A. baumannii 25	0.187	0.25	0.75	0.375	0.5	0.25
A. baumannii 26	0.375	0.375	0.25	0.375	0.187	0.375
S. aureus 6* *A. baumannii* 1	0.5	0.5	0.375	0.375	0.75	1.25
S. aureus 7* *A. baumannii* 1	0.125	0.375	0.093	0.125	0.375	1
S. aureus 22* *A. baumannii* 1	0.187	0.25	0.375	0.75	0.125	0.25
S. aureus 6* *A. baumannii* 8	0.25	0.25	0.125	0.375	0.75	0.75
S. aureus 7* *A. baumannii* 8	0.187	0.125	0.093	0.125	0.375	0.125
S. aureus 22* *A. baumannii* 8	0.093	0.375	0.125	0.25	0.75	0.25
S. aureus 6* *A. baumannii* 20	0.75	0.5	0.25	0.125	0.125	0.25
S. aureus 7* *A. baumannii* 20	0.187	0.093	0.75	0.25	0.5	0.25
S. aureus 22* *A. baumannii* 20	0.125	0.375	0.375	0.125	0.093	0.125

Abbreviation: GM, gentamicin; IMP, imipenem; FUC, fucoidan; FIC, fractional inhibitory concentration; FBC, fractional bactericidal concentration

Similarly, the combination of imipenem and fucoidan yielded values of 0.47, 0.46, and 0.37.

Additionally, we evaluated the optimal synergistic concentrations using the FBECi. For gentamicin and imipenem, the geometric mean values were 0.98, 0.69, and 0.24. For gentamicin and fucoidan, they were 0.55, 0.58, and 0.36. Lastly, for imipenem and fucoidan, the values were 0.52, 0.48, and 0.39 across the same isolates. Further details of these findings are presented in Table 8.

FE-SEM

The synergistic potential of gentamicin, imipenem, and fucoidan in eradicating or neutralizing dual-species biofilms of *S. aureus* and *A. baumannii* was evaluated using FE-SEM. The results demonstrated that the combination of fucoidan with antibiotics at their FBIC concentrations had a significant degradative effect on the biofilm structures. Specifically, the synergy between fucoidan and the antibiotics at these concentrations led to substantial disruption of the dual-species biofilm, causing bacterial lysis and a marked reduction in the overall biofilm biomass. The cohesive biofilm structures were broken down into smaller, less organized fragments. Moreover, the combination of gentamicin and imipenem also showed a strong synergistic effect, leading to the reduction of large biofilm biomass, bacterial lysis, and the formation of detached bacterial cells (Fig. 3).

Activity of synergistic effects on the biofilm encoding genes

Expression levels of the *icaA* gene in *S. aureus* and the *bap* gene in *A. baumannii* were evaluated after 24-hour exposure to 1/2 MBIC concentrations of gentamicin combined with imipenem, gentamicin combined with fucoidan, and imipenem combined with fucoidan. In single-species biofilms, *icaA* expression was downregulated by 2.98- to 1.52-fold, and *bap* expression by 3.15- to 1.94-fold. For dual-species biofilms, *icaA* in *S. aureus*

Table 7 Ranges of MBIC and MBEC for gentamicin, imipenem, and fucoidan

Strain	GM (μg/mL)		IMP (μg/mL)		FUC (μg/mL)	
	MBIC	MBEC	MBIC	MBEC	MBIC	MBEC
S. aureus 3	128	512	128	512	500	1000
S. aureus 6	64	256	128	256	250	500
S. aureus 7	128	512	64	256	250	1000
S. aureus 10	32	256	64	128	125	250
S. aureus 13	64	512	32	128	62.5	250
S. aureus 16	32	512	128	256	62.5	125
S. aureus 20	16	128	64	128	250	500
S. aureus 22	32	256	128	512	250	500
S. aureus 28	128	512	32	128	125	250
S. aureus 30	64	128	32	128	62.5	125
A. baumannii 1	256	512	128	256	62.5	125
A. baumannii 3	128	512	64	128	31.2	62.5
A. baumannii 4	128	512	128	256	250	500
A. baumannii 8	64	256	32	64	62.5	125
A. baumannii 13	128	512	16	64	31.2	62.5
A. baumannii 17	64	256	32	256	125	250
A. baumannii 20	256	512	64	256	500	1000
A. baumannii 22	128	256	32	128	62.5	125
A. baumannii 25	128	256	32	128	125	125
A. baumannii 26	64	256	64	256	250	500
S. aureus 6* *A. baumannii* 1	128	512	128	512	125	125
S. aureus 7* *A. baumannii* 1	64	128	32	64	62.5	125
S. aureus 22* *A. baumannii* 1	32	256	64	128	31.2	62.5
S. aureus 6* *A. baumannii* 8	16	32	32	64	62.5	62.5
S. aureus 7* *A. baumannii* 8	16	32	8	32	31.2	62.5
S. aureus 22* *A. baumannii* 8	8	32	32	64	62.5	250
S. aureus 6* *A. baumannii* 20	32	128	16	32	250	500
S. aureus 7* *A. baumannii* 20	16	64	16	32	31.2	62.5
S. aureus 22* *A. baumannii* 20	4	8	8	64	15.6	31.2

Abbreviation: GM, gentamicin; IMP, imipenem; FUC, fucoidan; MBIC, minimum biofilm inhibitory concentration; MBEC, minimum biofilm eradication concentration

decreased by 5.92- to 3.64-fold, while *bap* in *A. baumannii* was reduced by 5.78- to 4.17-fold. With gentamicin and fucoidan, *icaA* was downregulated by 3.32- to 1.90-fold and *bap* by 2.86- to 1.79-fold in single-species biofilms. In dual-species biofilms, *icaA* decreased by 6.12- to 4.20-fold in *S. aureus*, and *bap* by 5.68- to 3.79-fold in *A. baumannii*. After treatment with imipenem and fucoidan, *icaA* was downregulated by 3.1- to 1.72-fold, and *bap* by 3.12- to 1.68-fold in single-species biofilms. In dual-species biofilms, *icaA* decreased by 5.53- to 3.75-fold in *S. aureus*, and *bap* by 6.87- to 4.48-fold in *A. baumannii*. ANOVA showed significant differences in gene expression between single-species and dual-species biofilms ($p<0.05$) (Fig. 4).

Cytotoxicity of fucoidan

Cytotoxicity assessments revealed that fucoidan concentrations of 1000, 500, and 250 μg resulted in cytotoxicity levels of 78.3%, 42.1%, and 11%, respectively, in HSF-PI 16 cells. Importantly, when fucoidan was combined with the antibiotics imipenem (32 and 64 μg) and gentamicin (32 and 64 μg) at optimal synergistic concentrations of 125, 62.5, 32.1, 15.6, and 7.8 μg, no cytotoxic effects were detected in HSF-PI 16 cells. Furthermore, statistical analysis using a t-test indicated no significant differences in survival rates between the fucoidan treatments at these concentrations and the control sample ($p=0.083$).

Discussion

The DFUs represent one of the most severe complications of diabetes mellitus, often leading to chronic infections, delayed wound healing, and in extreme cases, limb amputation [42]. Among the microorganisms commonly involved in DFU infections, *S. aureus* and *A. baumannii* are particularly problematic due to their ability to form biofilms and develop resistance to antibiotics [9]. Biofilms, a structural mode of bacterial growth, significantly enhance bacterial survival and protect the organisms from antimicrobial agents, making infections exceedingly difficult to treat [43]. The increasing prevalence of MDR strains further exacerbates this challenge, emphasizing the urgent need for alternative therapeutic strategies [43].

Table 8 The optimal synergistic concentrations of gentamicin-imipenem, gentamicin-fucoidan, and imipenem-fucoidan against biofilm condition

Strain	FBIC-indices (GEN+IMP)	FBEC-indices (GEN+IMP)	FBIC-indices (GEN+FUC)	FBEC-indices (GEN+FUC)	FBIC-indices (IMP+FUC)	FBEC-indices (IMP+FUC)
S. aureus 3	1.5	1.25	0.75	1.25	1.25	1.5
S. aureus 6	1	1	0.375	0.75	0.75	0.75
S. aureus 7	0.375	0.75	0.375	0.5	0.5	0.75
S. aureus 10	0.25	0.5	0.25	0.5	0.25	1
S. aureus 13	0.75	1.25	0.75	0.5	0.75	1
S. aureus 16	0.75	1.25	1.25	1.5	1.25	0.75
S. aureus 20	1.25	1.25	0.5	0.125	0.25	0.125
S. aureus 22	1.5	1.5	0.5	0.25	0.125	0.125
S. aureus 28	1.25	1.25	0.75	1.25	1.5	1.25
S. aureus 30	0.75	0.5	0.125	0.375	0.125	0.125
A. baumannii 1	0.75	0.75	0.5	0.125	0.5	0.75
A. baumannii 3	0.375	0.5	0.5	1	0.75	0.75
A. baumannii 4	1	1	0.125	0.5	0.75	0.5
A. baumannii 8	0.375	1.25	0.75	1.25	1.5	1.25
A. baumannii 13	0.75	1.25	0.5	1	0.125	0.5
A. baumannii 17	0.75	0.375	0.375	0.125	0.375	0.125
A. baumannii 20	1.25	1.25	0.75	1.25	0.5	0.75
A. baumannii 22	0.75	1	0.5	0.75	0.125	0.75
A. baumannii 25	0.375	0.375	0.5	0.5	0.75	0.5
A. baumannii 26	0.25	0.25	0.375	1	0.5	0.125
S. aureus 6* *A. baumannii* 1	0.75	0.75	0.5	0.375	0.75	0.5
S. aureus 7* *A. baumannii* 1	0.5	0.75	0.125	0.25	0.5	0.75
S. aureus 22* *A. baumannii* 1	0.375	0.093	0.125	0.125	0.25	0.25
S. aureus 6* *A. baumannii* 8	0.125	0.187	1	1.25	1	0.75
S. aureus 7* *A. baumannii* 8	0.375	0.5	1	1.5	1.5	1.25
S. aureus 22* *A. baumannii* 8	0.187	0.187	0.25	0.375	0.25	0.5
S. aureus 6* *A. baumannii* 20	0.125	0.187	0.75	0.25	0.093	0.125
S. aureus 7* *A. baumannii* 20	0.093	0.125	0.125	0.5	0.375	0.5
S. aureus 22* *A. baumannii* 20	0.093	0.125	0.093	0.125	0.125	0.093

Abbreviation: GM, gentamicin; IMP, imipenem; FUC, fucoidan; FBIC, fractional biofilm inhibitory concentration; FBEC, fractional biofilm eradication concentration

Regrettably, a significant proportion of the *S. aureus* strains isolated in our study demonstrated resistance to methicillin, exceeding the rates reported in other studies [44–46]. Additionally, 77.41% ($n=24$) of the isolates were capable of forming strong and moderate biofilms. This indicates a significant association between this type of biofilm and the MDR of *S. aureus* isolates, a relationship we had previously demonstrated in *P. aeruginosa* studies [47]. Additionally, all isolates exhibited susceptibility to two antibiotics, linezolid and vancomycin. This observation underscores the continued efficacy of these drugs as preferred choices for treatment [48–50]. In hospital settings, upon detecting *S. aureus* in diabetic wounds, vancomycin is typically prescribed promptly. However, administering intravenous vancomycin to hospitalized patients can result in considerable expenses due to extended hospital stays, staffing requirements, and potential adverse reactions [51]. Of particular concern is vancomycin-associated acute kidney injury, especially for diabetic patients who are already at a higher risk of kidney complications [52]. Furthermore, the emergence of vancomycin-resistant strains of *S. aureus* highlights the need to explore alternative treatment strategies [53]. Therefore, employing a combination of two antibiotics with synergistic effects offers a sensible approach to treatment. In this study, upon investigating the presence of *A. baumannii* in diabetic wounds, we discovered that all isolates were MDR, and 88.8% of the bacteria demonstrated the ability to form strong and moderate biofilms, a concerning finding. Additionally, given the weakened immune system of diabetic individuals, the likelihood of developing a mixed infection, particularly with *S. aureus*, is notably high, which can cause an increase in infection severity may occur [54]. This significantly diminishes the prospects of successful treatment.

In polymicrobial infections, inherent resistance mechanisms of pathogens can influence the efficacy of certain antibiotics, sometimes resulting in enhanced

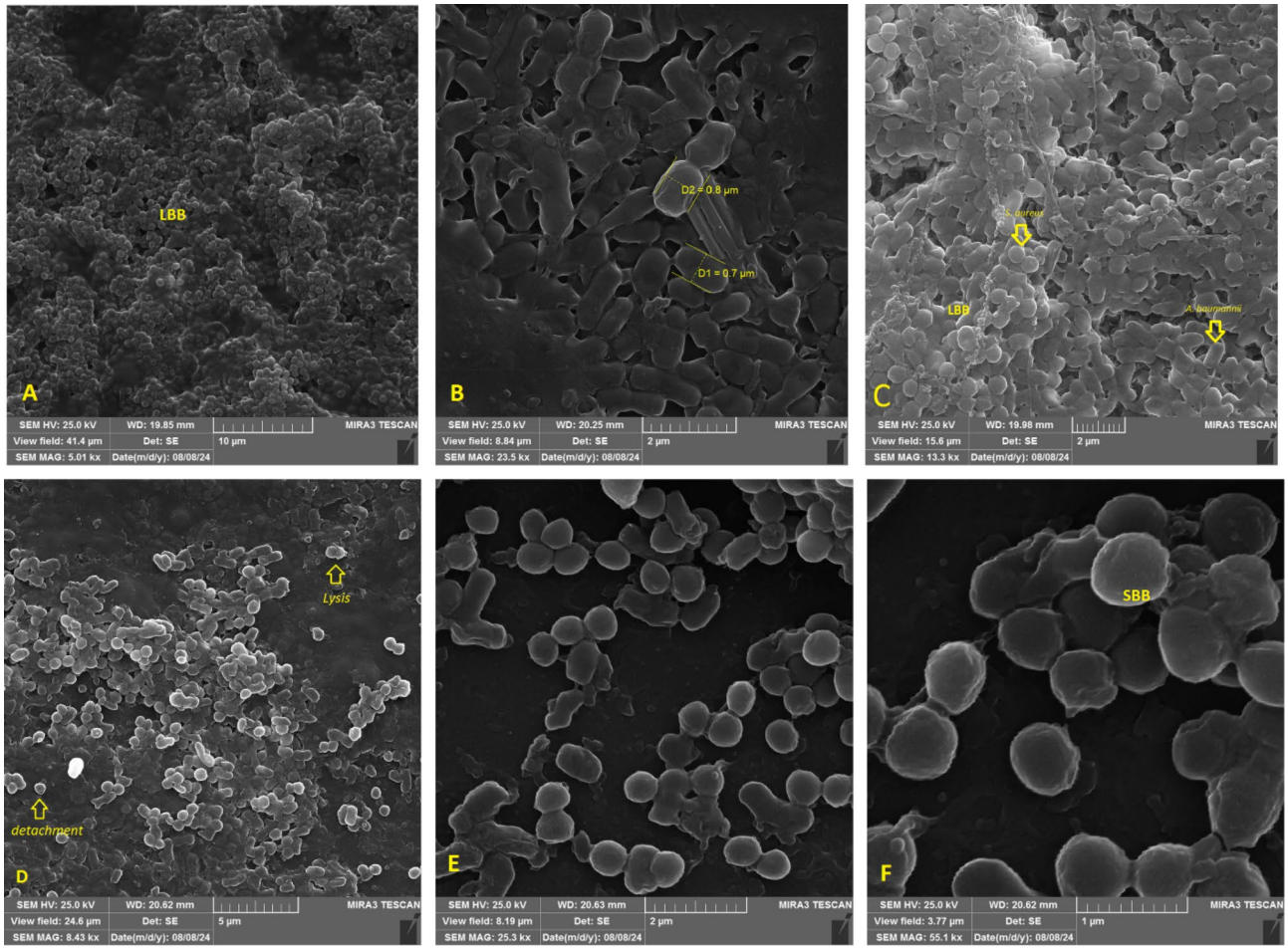

Fig. 3 Effect of combination gentamicin, imipenem, and, fucoidan on dual-species biofilms of *Staphylococcus aureus* and *Acinetobacter baumannii*. (**A**) single-species biofilm *S. aureus* (Untreated), (**B**) single-species biofilm *A. baumannii* (Untreated), (**C**) dual-species biofilm of *S. aureus* and *A. baumannii* (Untreated), (**D**) dual-species biofilm treated with 32 µg/mL gentamicin and 64 µg/mL imipenem, (**E**) dual-species biofilm treated with 32 µg/mL gentamicin and 31.2 µg/mL fucoidan, (**F**) dual-species biofilm treated with 31.2 µg/mL fucoidan and 64 µg/mL imipenem. LBB, large biofilm biomass; SBB, small biofilm biomass

performance during polymicrobial formation. Previous studies have shown that *Pseudomonas* spp. can alter the activity of antistaphylococcal agents by releasing endopeptidases, rhamnolipids, and 2-heptyl-4-hydroxyquinolone N-oxide [55, 56]. Our study found that co-culturing *S. aureus* and *A. baumannii* resulted in a lower geometric mean MIC and MBC for gentamicin, imipenem, and fucoidan compared to individual cultures, indicating increased sensitivity in co-culture. These findings align with previous research by Smith et al., which demonstrated that *S. aureus* enhances the sensitivity of *A. baumannii* to meropenem in co-culture [57]. However, Castellanos et al. observed no significant growth reduction or change in antibiotic susceptibility when co-culturing clinical strains of *A. baumannii* and *S. aureus* from a diabetic patient's skin and soft tissues [54]. This discrepancy might be due to the small sample size in the study by Castellanos et al. The observed reduction in resistance to imipenem, gentamicin, and fucoidan in our study can be attributed to several factors. Firstly, synergistic interactions between the bacterial species might enhance antibiotic susceptibility through metabolic interactions, gene expression changes, or alterations in the bacterial cell envelope [58]. Secondly, resource competition in the co-culture environment might induce physiological changes, such as metabolic shifts or altered growth rates, making the pathogens more susceptible to antibiotics [59]. Lastly, bacterial communication via quorum sensing and signaling molecules might affect antibiotic susceptibility by influencing the production and perception of these molecules in co-culture conditions [60, 61]. Moreover, the reduced geometric mean ratio of MBC to MIC in the co-culture of *S. aureus* and *A. baumannii* with imipenem, gentamicin, and fucoidan suggests an increase in the bactericidal potency of these antibiotics compared to their efficacy in individual cultures. This enhanced bactericidal effect underscores the potential benefits of

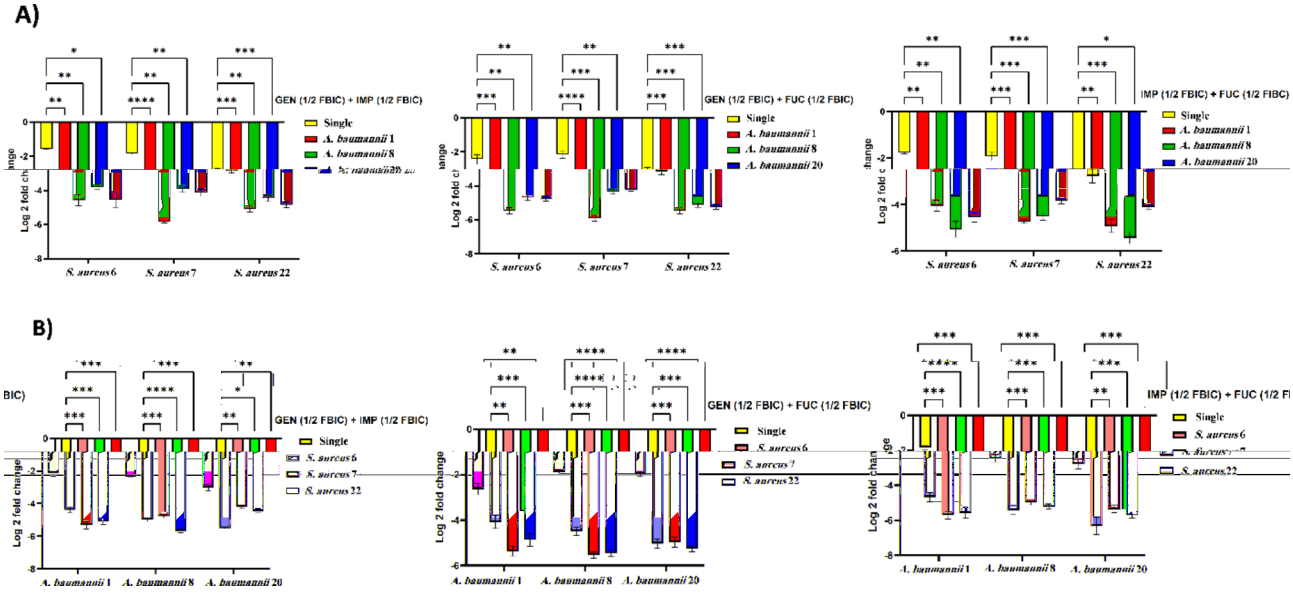

Fig. 4 Reduction in expression of biofilm-related genes *icaA* (**A**) in *Staphylococcus aureus* and *Bap* (**B**) in *Acinetobacter baumannii* when exposed to sub-FBIC of gentamicin, imipenem and fucoidan (GEN, gentamicin; IMP, imipenem, FUC, fucoidan; mean ± SD, $n = 3$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$)

considering polymicrobial interactions in antibiotic treatment strategies.

Rewrite the following text:

In our study, based on OD values, we observed that 16.6% (139/837) of the various conditions for the tested isolates were capable of forming moderate to strong dual-species biofilms. Notably, certain strains of *S. aureus* (e.g., *S. aureus* 2, *S. aureus* 10, and *S. aureus* 26) demonstrated an inability to form dual-species biofilms when paired with any of the *A. baumannii* isolates. However, this pattern was not seen with *A. baumannii*, suggesting that *S. aureus* may have a more substantial role in influencing dual-species biofilm formation compared to *A. baumannii*. It is also noteworthy, as demonstrated in previous studies, that when *S. aureus* and *A. baumannii* were co-cultured, there was no significant reduction in the growth of either clinical strain, indicating that both bacteria can coexist within the same infection site [54, 62]. This coexistence may further complicate treatment strategies and contribute to the persistence of infections in clinical settings.

In dual-species biofilms, *S. aureus* showed a marked increase in susceptibility to imipenem and gentamicin. Prior studies have underscored the role of siderophores produced by *A. baumannii* in increasing *S. aureus* susceptibility to various antibiotics, offering a potential explanation for the observed effect in our study [63]. Moreover, treatment of dual-species biofilms containing *S. aureus* and *A. baumannii* with broad-spectrum imipenem and gentamicin led to nearly fivefold lower inhibitory concentrations and eradication of both bacteria compared to monoculture treatment. This phenomenon mirrors findings from previous research indicating reduced *S. aureus* resistance to aminoglycosides in dual-species biofilms with *P. aeruginosa* [64]. Additionally, our investigation revealed that *S. aureus* impacts the susceptibility of *A. baumannii* biofilm-embedded cells to antimicrobials, especially aminoglycosides. However, the precise mechanism underlying the enhanced susceptibility of *A. baumannii* remains uncertain, suggesting the involvement of a specific metabolite produced by *S. aureus*, although the exact active molecule has yet to be identified. Additionally, in our study, the anti-biofilm effect of fucoidan was observed against both *A. baumannii* and *S. aureus*, in both single-species biofilm and dual-species biofilms. This finding aligns with the results of Chmit et al., who demonstrated the anti-biofilm activity of fucoidan against Gram-positive bacteria (*S. aureus*, *Enterococcus faecalis*, and *S. epidermidis*) as well as two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) [65]. Furthermore, Jun et al. also reported an anti-biofilm effect of fucoidan on dental plaque bacteria, specifically targeting biofilms formed by *Streptococcus mutans* and *S. sobrinus* [66].

The analysis of the synergistic effects of gentamicin, imipenem, and fucoidan against *S. aureus* and *A. baumannii* revealed distinct outcomes based on the bacterial state. When these bacteria were in their planktonic form and cultured individually, the geometric mean FICi and FBCi values generally indicated additive effects, with values between 0.5 and 1.0. However, when the bacteria were co-cultured, the FICi values dropped to ≤0.5, indicating a synergistic interaction [35]. This suggests that the combined effect of these antibiotics is significantly enhanced under co-culture conditions. These findings

align with previous research by Lee et al., who observed synergistic effects of fucoidan when combined with ampicillin and gentamicin against oral pathogens [67]. Similarly, Choi et al. reported that fucoidan improved the effectiveness of oxacillin and ampicillin against MRSA [68]. The observed synergy in planktonic cultures extends to dual-species biofilms of *S. aureus* and *A. baumannii*, suggesting that the simultaneous administration of gentamicin and imipenem, in the presence of fucoidan, could improve the treatment outcomes for infections involving dual-species biofilms.

The synergistic potential of gentamicin, imipenem, and fucoidan in eradicating or neutralizing dual-species biofilm of *S. aureus* and *A. baumannii* was assessed through the use of FE-SEM. The experimental outcomes revealed that when fucoidan was combined with antibiotics at the concentration of FBICs, a remarkable and potent degradative effect was observed in biofilm structures. According to the results, fucoidan and antibiotics at the concentration of FBICs produced high destruction effects on the dual-species biofilm layer and interbacterial biofilm and lysis of bacteria and it reduced the large biofilm biomass and turned it into small biofilm biomass and destroyed the cohesive structures of the biofilm. Synergism of gentamicin-imipenem also decreased large biofilm biomass, caused bacterial lysis, and led to the formation of detached bacteria (Fig. 3).

To explore the anti-biofilm efficacy of the synergistic combination of gentamicin, imipenem, and fucoidan and to elucidate its underlying mechanisms, we conducted real-time PCR analysis to evaluate the expression of biofilm-associated genes in both single-species biofilm and dual-species biofilms. Our findings revealed a significant downregulation of the *icaA* gene in *S. aureus* and the *bap* gene in *A. baumannii* within dual-species biofilms compared to single-species biofilms. This observation is consistent with the study by Mani et al., which also demonstrated that their treatment not only exhibited antibiofilm and antimicrobial effects against *Proteus vulgaris* and *Salmonella enterica* but also effectively suppressed the expression of biofilm-related genes in these pathogens [69].

Conclusion

Our investigation into the combined effect of gentamicin, imipenem and fucoidan revealed synergistic interactions in dual-species biofilms of *S. aureus* and *A. baumannii*, presenting promising implications for treatment efficacy. Leveraging the synergistic effects of these antimicrobials holds the potential to improve treatment outcomes for dual-species biofilm infections. These findings highlight the intricate interplay between microbial interactions, antibiotic susceptibility, and biofilm formation in DFUs, emphasizing the necessity of tailored therapeutic approaches to effectively manage these complex infections. Prospective studies could center on exploring the precise antibacterial and anti-biofilm mechanisms of the gentamicin, imipenem and fucoidan combination via in vivo and molecular analyses. Furthermore, from a broader perspective, we propose that introducing antagonistic bacteria into preexisting monoculture biofilms could enhance their antimicrobial treatment efficacy. While our study demonstrated the synergy of interbacterial antagonism with antimicrobials using *S. aureus* and *A. baumannii* model system, we believe that numerous other bacteria from the normal body microflora could serve as antagonists against nosocomial pathogens. This suggests that microbial transplantation strategies could be explored to enhance the treatment of microbial infections.

Abbreviations

MDR	Multidrug-resistant
MRSA	methicillin-resistant S. aureus
DFU	diabetic foot ulcer
S.a	S. aureus
MSA	mannitol Salt Agar
TSA	trypticase soy agar
TSB	trypticase soy broth
BHI	brain heart infusion
MHB	Mueller-Hinton broth
PCR	polymerase chain reaction
OD	optical density
CFUs	colony-forming units
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
MBIC	minimum biofilm inhibitory concentration
MBEC	minimum biofilm eradication concentration
FICi	fractional inhibitory concentration index
FBCi	fractional bactericidal concentration index
FBICi	fractional biofilm inhibitory concentration index
FBECi	fractional biofilm eradication concentration index
SEM	Scanning electron microscopy

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Author contributions

MN performed all experiments and analyses, and also wrote the manuscript. MYA served as advisor. MT, MB and FN contributed as a supervisor and also in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The Ethics Review Board of the Hamadan University of Medical Sciences, Hamadan, Iran approved the present study (Ethical approval code: IR.UMSHA.REC.1401.809). Ethical Review Board approved informed consent taken from

all the participants, and all experiments were performed in accordance with relevant guidelines and regulations. We reported our findings according to the STROBE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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